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Progress in Development of Immunocontraceptive Vaccines for Permanent Non-surgical Sterilization of Cats and Dogs

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Contents

Each year, millions of cats and dogs are euthanized worldwide. There are insufficient resources to control shelter animals in developed countries, as well as feral cat and wild dog population levels, with current surgical sterilization techniques. Thus, population control of these animals will likely depend on the development of new non-surgical methods for cat and dog sterilization. One promising area of research is the development of contraceptive vaccines, or immunocontraceptives. In this article, previous approaches aimed at developing contraceptive vaccines will be reviewed, with a focus on those most related to sterilization of cats and dogs. There are a number of steps in reproduction that have been, or could be, targeted by the immune system, and the advantages and obstacles for inducing immunity to each of these will be discussed. Our current understanding of how these vaccines cause sterility, and our current ability to dissect these mechanisms in cats and dogs, also will be discussed.

Introduction

Domestic cats have established feral colonies on every continent except Antarctica. Feral cats are prolific hunters and constitute a threat to biodiversity in many areas. Dogs also have spread throughout the world and constitute a danger to human public health through the spread of rabies virus. In the United States alone, millions of dogs and cats are euthanized each year because of an insufficient number of adopters, and population control through surgical sterilization is inadequate, primarily because of the cost as well as logistic problems of trapping feral cats and dogs for sterilization.

Attempts to sterilize cats and dogs with contraceptive vaccines date back to the 1980s. Although advances have occurred, there are no vaccines that are sufficiently developed for practical use. The problems with these vaccines are varied and include: (i) the need for repeated booster vaccinations to achieve effect, rather than a one-time treatment; (ii) variability in immune responses between individual animals; (iii) recurrence of fertility as immune responses wane over time; and (iv) side effects induced by the use of adjuvants. In this review, some important differences in the design of various studies will be highlighted, as well as a lack of reagents to dissect the immune mechanisms that lead to sterilization.

Self-tolerance and Adjuvants

The mammalian immune system has mechanisms in place to ensure that T cells and antibody-producing B cells do not respond to so-called self-antigens. The processes that ensure self-tolerance occur in the thymus for developing T cells, the bone marrow for developing B cells and the periphery for both mature T cells and mature B cells. Under normal physiological conditions, this self-tolerance prevents T cells and B cells from causing autoimmunity. In the case of contraceptive vaccines, self-tolerance must be broken to cause infertility. This is achieved with the help of adjuvants, substances that induce the immune system to react to a specific component of the reproductive system.

There are three main categories of contraceptive vaccines reported in the literature: (i) subunit vaccines; (ii) DNA vaccines; and (iii) recombinant vaccine vectors. These have important differences in their design, and each breaks tolerance via separate mechanisms.

Subunit vaccines are generally the simplest in design. These include an antigen as well as an exogenous (i.e. added) adjuvant to activate the innate immune system and induce effective antibody and T cell responses. For example, GonaCon™ [United States Department of Agriculture s (USDA) Wildlife Services (WS) National Wildlife Research Center (NWRC), Fort Collins, Colorado, USA] includes gonadotropin-releasing hormone (GnRH), coupled to a carrier protein, as the antigen, and a purified fraction of *Mycobacterium avium* as the adjuvant.

DNA vaccines are plasmid DNA, purified from *E. coli*., that encode recombinant antigen under a promoter that allows expression in the target species. Bacterial DNA has a higher proportion of CpG dinucleotides than mammalian DNA, and bacteria hypomethylate these sequences, compared to mammals. The presence of these hypomethylated CpG sequences leads to activation of innate immunity via Toll-like receptor 9 (TLR9), a pro-inflammatory receptor that potently activates innate and adaptive immunity.

Recombinant vaccine vectors are attenuated microorganisms, most commonly viruses or bacteria, that have been modified to express the desired antigen. These, and other pathogens, contain endogenous adjuvants and often are robust activators of the immune system. These endogenous adjuvants include a variety of pathogen-associated molecular patterns (e.g. TLR ligands) as well as activation of cell damage and cell death pathways. Elucidation of these pathways is currently an area of intense research activity but they are not all clearly defined, and their ability to activate adaptive immunity often is more poorly understood than their activation of innate immunity. However, it is clear that many or most pathogens activate multiple pathways, and together, these can result in strong adaptive immune responses.

Reproductive Targets

To be ideal, the target of a contraceptive vaccine must meet multiple requirements. First, it must be absolutely essential for reproduction. Second, it must have no important physiological function other than reproduction. Third, it is preferable that the target be essential for both female and male reproduction, or else a separate product would need to be developed for each sex. Fourth, the product also should eliminate unwanted sex-related behaviours. Finally, it is preferable that the target be extracellular, because antibodies are very poor at targeting intracellular antigens under normal physiological conditions.

There are several reproductive proteins that meet these criteria, with GnRH and zona pellucida (ZP) proteins studied most often and in the largest number of species. In theory, the sex steroids (e.g. progesterone, oestradiol and testosterone) also could be useful contraceptive vaccine targets. Indeed, measurement of these relies on immunoassays that utilize antibodies that are specific to individual sex steroids. However, because sex steroids share common precursors, many antibodies would not be specific to the intended hormone only, likely resulting in unwanted side effects.

GnRH

GnRH is well studied as a target for contraceptive vaccines and is the basis for the GonaCon™ vaccine. Because GnRH induces the release of gonadotropins from the pituitary, it is essential in both males and females. GnRH is a 10-amino acid peptide and thus can be readily synthesized.

However, use of GnRH as an immunocontraceptive has disadvantages. Because of its length, it is poor at activating CD4 T cells, which help achieve optimal B cell activation and antibody production. Therefore, it must be conjugated to a carrier protein capable of activating CD4 T cells. In the case of GonaCon™, GnRH is conjugated to keyhole limpet haemocyanin (KLH). Keyhole limpet haemocyanin is the oxygen-carrying metalloprotein of the giant keyhole limpet, analogous to haemoglobin, and is very large. Thus, it has numerous CD4 T cell epitopes.

GonaCon™ and other GnRH vaccines have been tested in over a dozen species, including cats and dogs, and have provided encouraging results. In one study on male cats (Levy et al. 2004), two-thirds of the vaccinated cats were classified as strong responders (antibody titre $>1:32\,000$) and had prolonged loss of or decrease in testosterone, scrotal volume, semen volume and sperm counts. In a study of female cats (Levy et al. 2011), two of 15 females had antibody titres of 1 : 32 000 and 13 of 15 had titres $>$ 1 : 32 000. Some cats had waning antibody titres over time, although 11 of 15 (73%) females were classified as long-term responders and maintained high antibody titres for at least 24 months. A breeding trial demonstrated that after 3 years, 53% of females remained infertile, with 40% remaining infertile at 4 years.

These data demonstrate that antibodies to GnRH are capable of causing sterility in both male and female cats. However, they also highlight the difficulty in achieving permanent, or even long-term, sterility with a single-dose injection. The high antibody titres required to achieve and maintain sterility in these studies may be due to the biology of GnRH release. GnRH is released from the hypothalamus in pulsatile bursts and only travels a very short distance in the blood stream to reach the pituitary gonadotrophs that respond to GnRH. Thus, antibodies have a small window of opportunity to neutralize GnRH and must be present at a very high concentration to be effective.

GnRH receptor

The gonadotrophs of the anterior pituitary express GnRH receptor (GnRH-R) and respond to GnRH stimulation by releasing the gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which act on the gonads. Gonadotrophs are thought to be the only cell type that expresses GnRH-R and that is capable of responding to GnRH stimulation; thus, these cells are also essential for reproduction in both females and males. In addition, these cells are thought to be non-renewable, which should make them a good target for permanent sterilization.

There are no studies where GnRH-R has been targeted as a contraceptive vaccine, but the feasibility of eliminating these cells with cytotoxin conjugates has been examined. A GnRH analogue, which binds to GnRH-R, was linked to a cytotoxin that was then administered to dogs (Sabeur et al. 2003; Ball et al. 2006). After two to three treatments, most dogs had a drop in testosterone, basal LH, LH following GnRH stimulation and testis volume. Although none of the dogs had a loss of testosterone after a single treatment, these studies demonstrated that gonadotroph ablation is a viable option for sterilization. Furthermore, none of the dogs that lost testosterone production had a subsequent rebound of serum testosterone over the subsequent 4–12 months.

One major disadvantage of GnRH-R as a contraceptive vaccine target is that it is a member of the seven-transmembrane, G-protein-coupled receptor (GPCR) family. Integral membrane proteins, such as GPCR proteins, require a lipid membrane for their stability and are thus not easily purified. Furthermore, purification requires detergents, which can be reactogenic in vaccines and generally are avoided in vaccine formulations.

FSH and LH

GnRH induces secretion of FSH and LH from gonadotrophs into the blood, where they travel to and act on the gonads of females and males. Follicle-stimulating hormone and LH are dimeric protein hormones, composed of a shared alpha subunit and unique beta subunits, that could be targeted by a contraceptive vaccine, similarly to GnRH. Unfortunately, thyroid-stimulating hormone (TSH) also utilizes the same alpha subunit. This makes targeting of FSH and LH problematic. Thyroid-stimulating hormone has many important physiological functions, including control of metabolism, and antibodies that interact with the alpha

subunit of FSH and LH would also likely recognize the alpha subunit of TSH.

FSH-R and LH-R

Mice that genetically lack the LH receptor (LH-R) have been created, and both sexes are sterile (Ascoli et al. 2002), as are humans with genetic defects in LH-R. Follicle-stimulating hormone signalling through FSH receptor (FSH-R) regulates expression of LH-R, suggesting that FSH-R also may be essential for reproduction.

Similar to GnRH-R, LH-R and FSH-R are GPCRs that are integrated into the cell membrane. While purification of these is more difficult than purification of secreted proteins, one group has purified LH-R from bovine luteal cells using Triton X-100 detergent and was able to remove the detergent while maintaining binding activity of the LH-R (Saxena et al. 2002, 2003). Following emulsification of the LH-R with a mycobacterial adjuvant and encasement in perforated silastic tubing, dogs (Saxena et al. 2002) or cats (Saxena et al. 2003) were vaccinated. All five vaccinated dogs made antibody responses to LH-R. This resulted in decreased progesterone that lasted from approximately 6 months to 1.5 years and was inversely correlated with the antibody titre. Similar results were observed in cats. While these studies did not result in permanent sterilization, they demonstrated that immune responses to LH-R can result in loss of progesterone, and presumably fertility, as long as antibody titres remain elevated.

Zona pellucida proteins

Contraceptive vaccines containing ZP proteins have been studied since the 1970s, when they first tested in horses. There are three ZP proteins, ZP1, ZP2 and ZP3 (ZP-A, ZP-B and ZP-C in cats). They are extracellular glycoproteins expressed only in females, in the ovaries, and form a matrix around the developing follicle. After ovulation, the matrix continues to surround the egg and forms the sperm docking site, initiating the acrosome reaction.

Porcine ovaries are available from abattoirs, and porcine ZP (pZP) is the most studied ZP in contraceptive vaccines. Vaccination against ZP proteins causes non-permanent sterilization of horses and has been tested in numerous other species. Although the first report on the use of ZP in cats used a five-vaccine regimen, the results were promising, with only one of five female cats becoming pregnant (Ivanovo et al. 1995). However, other studies have been much less successful. A single vaccination with pZP, administered with either Freund's complete adjuvant (FCA) or aluminium adjuvant, did not alter follicle development or fecundity of female cats (Gorman et al. 2002). This study suggested that these results were attributed to poor cross-reactivity between porcine ZP and feline ZP proteins. A later study, using cow, mink or ferret ZP proteins to immunize cats, found that these also induced antibodies that were poorly cross-reactive with feline ZP (Levy et al. 2005). A third study found that a three-dose regimen of pZP in cats did not alter ovarian follicle development, despite the presence of anti-pZP antibodies, and that the FCA and alum adjuvants resulted in severe adverse reactions (Munson et al. 2005).

Studies with pZP have also been conducted in female dogs, resulting in infertility (Mahi-Brown et al. 1982, Mahi-Brown et al. 1985). Serum antibodies from sterilized dogs reacted to dog ovaries and were able to prevent fertilization *in vitro*. However, often this occurred only after three to six vaccines were administered (Mahi-Brown et al. 1985). Furthermore, loss of fertility was sometimes associated with abnormal oestrous cycling, indicating that antibodies also may have responded to other ovarian proteins.

The ZP proteins are poorly conserved between species and represent an important barrier for prevention of cross-species fertilization. So perhaps it is not surprising that while pZP elicits antibodies that work well in ungulates such as deer and horses, they are less effective in more distant species such as cats and dogs. Cat and dog ovaries are not readily available in large quantities for purification of fZP and canine ZP (cZP), and it may be cost-prohibitive to express and purify these. As an alternative to protein purification, it was shown that if fZP is expressed from a DNA vaccine, incomplete contraception can be achieved in cats (Eade, 2009). This circumvents the problems of poor cross-reactivity between ZP from different species as well as the necessity of expressing and/or purifying fZP and cZP.

One species where ZP contraception has achieved notable success is in mice. An Australian group, attempting to alleviate the periodic mouse plagues that occur there, expressed mouse ZP proteins from the herpesvirus mouse cytomegalovirus (MCMV) (Lloyd et al. 2003). Vaccination of mice with MCMV expressing mouse ZP3 (MCMV-mZP3) caused sterility in vaccinated female mice. This effect required only a single vaccination, resulting in long-lived antibody responses. Presumably, this occurred, at least in part, because herpesviruses establish latency and are never completely eliminated from the host and then undergo periodic reactivation. The effect lasted more than 1 year, but the relatively short lifespan of mice prevents a truly long-term study from being conducted.

A similar approach has been attempted to create a contraceptive vaccine for foxes using canine herpesvirus (CHV). In that regard, CHV was modified to create a bacterial artificial chromosome (BAC) version of the virus, which greatly improves the ease of mutating the viral genome (Strive et al. 2006). Fox ZP3 then was expressed from the CHV and used to vaccinate foxes. Unfortunately, the study was unable to demonstrate any replication of the virus, and foxes did not produce antibodies to fox ZP3 or to another marker, enhanced green fluorescent protein (EGFP). The lack of replication and inability to induce antibodies may have been due either to the use of a dog-specific virus in foxes or to the removal of the CHV thymidine kinase (TK) gene. In addition, the decision not to remove the large BAC cassette may have decreased viral fitness, for example, through inefficient packaging.

Feline Herpesvirus-1 as a recombinant vaccine vector

The approach recently undertaken by our group has been to build on these findings with herpesviruses to see whether a similar approach might work in cats and dogs. We are in the process of creating an attenuated strain of feline herpesvirus-1 (FHV-1) that expresses recombinant feline ZP (fZP) and/or other proteins required for reproduction. The goal is to develop a vaccine that, through attenuation, does not cause disease in vaccinated cats, but is capable of eliciting antibodies that result in sterilization. Future work will evaluate a similar vaccine for dogs using CHV.

There are several major advantages to working with herpesviruses. First, the pathology and epidemiology of FHV-1 in cats and CHV in dogs are relatively well defined. Both of these are thought to be quite species specific, infecting only felides or canids, respectively, although the exact host range is not completely defined. It is documented that they are unable to infect cells from humans and other primates, rodents or rodent cells, as well as many other animals outside the order *Carnivora* (Povey 1979). In fact, FHV-1 is unable to infect canids, and CHV is unable to infect felids, despite their close evolutionary history.

We have studied the immunogenicity of FHV-1 in some detail. To determine how immunogenic FHV-1 is, we have established an ELISA that measures the relative abundance of anti-FHV-1 antibodies in cat serum. The assay is sensitive enough to routinely detect antibodies in serum diluted 1 : 100 000 or more (Fig. 1). The assay also has a large dynamic range, allowing us to compare cats whose serum antibody titres vary by 100-fold or more.

An FHV-1 vaccine exists, derived from an attenuated strain of FHV-1 that is still replication competent, and this vaccine is incorporated into most if not all of the current trivalent and quadrivalent commercial feline vaccines. Multiple vaccinations with these formulations are recommended for cats, along with periodic booster vaccinations, and thus, client-owned cats are typically vaccinated repeatedly against FHV-1, which may account for these high titres. Our project will test whether a single dose of recombinant FHV-1, like the recombinant MCMV discussed previously, can induce high-titre, long-lived antibody responses to reproductive proteins that alter fertility in cats.

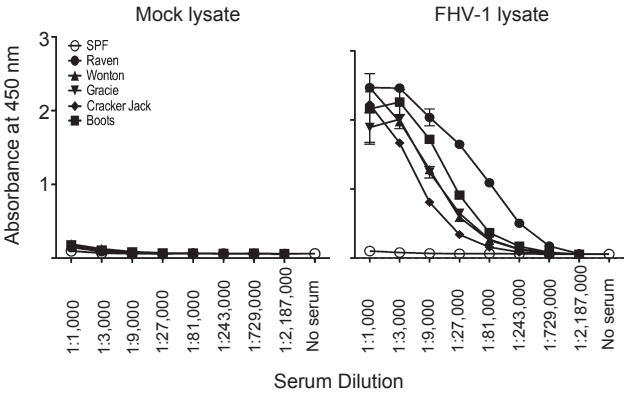

Fig. 1. Cats have high antibody titres to FHV-1. Lysates of Crandall-Reese feline kidney (CRFK) cells, either infected with FHV-1 or mock-infected, were coated onto ELISA plates. Plates were blocked and then incubated with serial dilutions of serum from client-owned cats. Antibodies to FHV-1 were detected with goat anti-cat IgG-Fc polyclonal antibody, conjugated to HRP, using TMB as a substrate. Serum from pooled specific pathogen-free (SPF) cats demonstrated the specificity of the assay

Immune Mechanisms

Very few studies of contraceptive vaccines have been reported in which the mechanism of contraception has been examined. For GnRH, it seems very likely that neutralization by antibodies is the primary or only immune mechanism. If vaccines are developed in the future that specifically target FSH or LH, antibody neutralization would presumably be the dominant mechanism of action for these as well.

It is also possible to target hormone receptors, including GnRH-R, FSH-R and LH-R. Indeed, an LH-R vaccine already has been tested in dogs and cats with some success (Saxena et al. 2002, 2003). In this case, it is possible that antibody could bind to LH-R and prevent binding of LH, thus preventing signalling. However, antibodies have other functions in addition to neutralization. These include target cell lysis through complement fixation and antibody-dependent cell-mediated cytotoxicity (ADCC), the latter of which can activate natural killer (NK) cells or neutrophils to lyse target cells. Furthermore, it is possible that T cells could target the cells expressing GnRH-R, FSH-R or LH-R. CD8 T cells can either kill cells directly, through perforin and granzyme B secretion or through FasL/Fas interactions, or can secrete cytokines, including IFNγ and TNFα. CD4 T cells, while not well known for killing, also can contribute to cell death through cytokine release and recruitment of other immune cells.

When mice were vaccinated against ZP3, ovarian histology showed that follicle development was interrupted (Lloyd et al. 2003). It was not determined, however, whether this was because of neutralizing antibody blocking ligand–receptor interactions, antibody-mediated complement fixation, ADCC or T cell killing of cells expressing LH-R.

The hypothalamus also could be susceptible to T cell attack if it were not for the protection of the blood–brain barrier (BBB). Unlike other blood vessels, those in the brain form tight junctions that prevent cells and macromolecules from leaving the blood vessels. T cells and antibodies thus cannot gain access to the hypothalamus. The anterior pituitary, where gonadotrophs reside, is outside of the BBB and thus is accessible to both T cells and antibodies.

In the example of ZP proteins, there is some evidence for the immune mechanisms leading to infertility. Originally, it was found that antibodies to ZP proteins can inhibit fertilization *in vitro,* and there is evidence that this also occurs *in vivo* (Barber and Fayrer-Hosken 2000). In mice, where there are a great number of tools available for dissection of the immune response, it is now becoming clear that while inhibition of sperm docking may occur, immune responses to ZP proteins also lead to depletion of late-stage follicles within the

ovary (Lloyd et al. 2003). There are several mechanisms that could lead to this. In the case of MCMV expressing ZP3, mice that have been genetically modified so that they are unable to make antibodies do not become sterile following vaccination with MCMV-ZP3 (Lloyd et al. 2010). While it could be argued that antibodies may contribute to the activation of T cells in the ovary, thus not directly causing loss of developing follicles, it was shown that transfer of immune serum from mice vaccinated with MCMV-ZP3 to otherwise untreated mice leads to transient infertility. This is strong evidence that antibodies alone are necessary and sufficient to mediate sterility in female mice.

Sophisticated tools are available for dissecting the immune response of mice, and the mechanisms that lead to infertility can be studied using genetic ablation or depletion of different cell types and effector functions, both through genetics and pharmacology. Unfortunately, similar tools are not available for cats, dogs or larger animals. For example, I am not aware of reagents that are capable of depleting CD4 T cells, CD8 T cells, B cells, complement or NK cells in either cats or dogs. Furthermore, it is possible that antibodies of different isotypes have varying degrees of activity in immune contraception, but there are few reagents available to measure the various isotypes of cat and dog antibodies.

Further development of these tools in the future would be helpful in performing studies that do not rely simply on observations and correlations, but allow true functional studies. Such studies could provide invaluable insight into the precise immunological mechanisms that make the most successful contraceptive vaccines work, as well determining why other vaccines are less successful. Knowledge of these mechanisms would then allow refinements to further improve the long-term prospects of contraceptive vaccines.

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Conflicts of interest

The authors declared that there are no conflicts of interest.

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